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(54) Title: DIAGNOSTIC AND THERAPEUTIC MEANS FOR PATHOLOGIES ASSOCIATED WITH ALPHA 2 SUBUNIT OF THE NA, K PUMP

(57) Abstract: A nucleic acid is described that comprises at least one segment of the gene encoding a functional segment of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in the diagnosis or treatment of pathologies associated with migraine or with alternating hemiplegia of the childhood. Appropriate diagnostic kits and methods to identify agonist or antagonist agents are also described.

DIAGNOSTIC AND THERAPEUTIC MEANS FOR PATHOLOGIES ASSOCIATED WITH ALPHA 2 SUBUNIT OF THE NA,K PUMP

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The present invention concerns diagnostic and therapeutic means for pathologies associated with alpha 2 subunit of the Na,K pump, as migraine and alternating hemiplegia of the childhood.

Migraine is characterized by headache attacks and is sometimes associated with autonomic nervous system dysfunctions and transient neurological symptoms (aura). The prevalence of migraine in the general population is 12%, and 20% of patients present with aura (1—5).

Although the mode of transmission is controversial (6), migraine shows strong familial aggregation (7). Several population-based and twin-based studies have indicated that genetic factors are implicated, particularly in migraine with aura (8, 9). Familial hemiplegic migraine (FHM) is a disabling neurological disease that manifests with aura and hemiparesis. It affects about 1/10,000 to 1/50,000 individuals and is transmitted as an autosomal dominant trait (10).

A gene associated with FHM1(MIM 141500) and encoding a neuronal calcium channel protein (CACNA1A) has previously been identified (11).

The PCT patent application WO98/55647 describes an indirect genotyping method for diagnosing hemiplegic migraine type 2 that concerns a wide region of 21 cM (centimorgan) of chromosome 1q21-23. The description does not identify any genes associated with the disease but suggests two candidate genes, GIRK3 (encoding a potassium channel protein) and CACNL1A6 (encoding a calcium channel protein).

The authors of the present invention have identified the gene associated with FHM2 (MIM 602481) that maps on chromosome 1q23 (12) and have shown that mutations in the alpha 2 subunit of the Na,K ATPase pump (ATP1A2) are responsible for the disease. The identified gene does not correspond to any genes or regions suggested in the previous technical documentation, particularly as regards the aforesaid patent application WO98/55647. The

authors have demonstrated that the identified missense mutations cause a loss-of-function of the major ion transport system. This has relevant implications for the origin of cortical spreading depression of neuronal disease and the development of migraine. It is the first demonstration that mutations in the Na,K pump are associated with genetic diseases. It should also be stressed that a study on GIRK3 and CACNL1A6 have not shown any mutations in these genes that could be correlated with migraine.
Furthermore, it is possible to study polymorphisms associated with said gene to correlate them as predisposing factors for common migraine.
Alternating hemiplegia of the childhood (AHC, OMIM 104290) is a rare syndrome (estimated prevalence 1 in 1.000.000), characterized by early onset of episodic hemi- or quadriplegia lasting minutes to days.
Mutation analysis in the ATP1A2 gene was performed by direct sequencing of all exons with the same primers used for amplification. An heterozygous mutation (1237 C->A) segregating with the disease in a AHC family and causing a threonine to asparagine replacement (T378N) was found. This mutation is not present in any of the unaffected members of the family and in 250 control chromosomes.
Hence, the object of the present invention is a nucleic acid comprising at least one segment of the gene encoding a functional portion or gene-regulating region of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in the diagnosis of pathologies associated with migraine or with the alternating hemiplegia of the childhood.
A further object of the invention is a nucleic acid comprising at lease one segment of the gene encoding a functional portion or gene-regulating region of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in genetic therapy for pathologies associated with migraine or with the alternating hemiplegia of the childhood.
A further object of the invention is a method to detect in an individual at least one mutation of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) located on chromosome1, associated with

migraine disorders or with the alternating hemiplegia of the childhood, which comprises the steps of: - collecting a sample containing a sufficient quantity of the individual's DNA or that is reproducible in culture; - isolating the DNA from the sample; - exponentially amplifying the DNA using as an oligonucleotide pair for the amplification reaction at least two oligonucleotides that are able to amplify at least one segment of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) or of a segment of the region regulating it; - detecting in at least one amplified segment any mutations compared with a healthy control. Preferably, the oligonucleotide pairs are:

17	AGTCCCTCTGACCTCCCTGAT	CCACTGTGCCATCACGATT
19	CTTCTGCTTCCTGCTCTGACC	ACACATGTGCGCTGTGTTTAC.

In an embodiment of the method of invention, the DNA exponential amplification phase is performed using oligonucleotide pairs that are able to amplify the entire encoding portion of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2). Preferably, the DNA exponential amplification phase to amplify the entire encoding portion of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) comprises the use of at least one of the following oligonucleotide pairs:

1 TGTTGCTTTGGCTTTCTCTGT	CTCCCTCACCCTCTAGACTGC
2+3 CCCCTCTCTTCCCTGACTCT	GCCTCTTTTGTTCCTTCCCTA
4 ATGGTGACTGGCTGGGTTG	CAGGGTTGGAGGACAGTCAC
5 AGCTGCCCCTTTAGGGTTG	ACCTTACAGCCTAGCCCAGAG
6 GAGACCAGCAGGAGAAGAAGG	AGACTCAACTGCTTGCTCTGG
7 TACAAGTGGCTCTGCCAGTCT	AGCCCTTCATCCTGACTATGG
8 CAGGAAATAGGATGGGACTGC	GTAGTGAGACCCTCCCCTGGT
9 ATCTCCGGCTTCAGCCTTAAC	TAATCCTATCCACCCCCTCTG
10+11 CTCCTGGTTCCCCCTCAT	TCCCTCTCTCTTCCTCTGTCC
12 GCGCTACCAAGACAAGTATGG	CTTGGGAATCCCCTTCTGAG
13 GAAGCCACTCTGCGGATCT	ACTGCAGCTCCTTGAACTCTG
14 GGAGGGGGATAAACCCTTAAT	GACGTGTTGATTAGGGCACAG
15 AGGGGTCAGCTGTCTCTGTC	GGTCCCTGCCTGTCATCTG

16	AAGGGGTTTCGTCCTCAAGT	TCAGTATCCTGCAAACCATCC
17	AGTCCCTCTGACCTCCCTGAT	CCACTGTGCCATCACGATT
18	TCATCTCCTACGTCCCTTCAA	AGCTGGGAAAAGAACCCTGT
19	CTTCTGCTTCCTGCTCTGACC	ACACATGTGCGCTGTGTTTAC
20	CCTCCGACACTCTCATCTGTC	CTGTGTGGGTTGGTGAGTGT
21	CTTCACCTGCCACCTCCTT	CCCCCGTATGACTACTCAGG
22	CGCTTTGAATGCTCCTTTATG	GAGGGAGGAGCTGGTGGT
23	GCCTCCTTTTAAGCTCATGCT	GCCTCATTATCTCTCCCCAAA

In an embodiment of the method of invention, the DNA exponential amplification phase is performed using oligonucleotide pairs that are able to amplify the regulating region of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2). Preferably, the DNA exponential amplification phase to amplify the regulating region of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) comprises the use of the following oligonucleotide pairs:

1_Pr	TTCCCCTCACTCCATCTCTG	GACCCCTGCTCTTTAGGGATA
2_Pr	GATTCAGGACCACTCCATCC	GGGAACAGTCAGAGGACAGG

In a preferred embodiment of the method of the invention, the detection phase of at least one amplified segment with any mutations compared with a healthy control is performed using direct sequencing or an SSCP method (single strand conformation polymorphism) (17) DHPLC or DGGE (denaturing gradient gel electrophoresis) (18) or other methods known to an expert from the field.

A further object of the invention is a diagnostic kit for pathologies associated with migraine or with alternating hemiplegia of the childhood which comprises: - at least a pair of oligonucleotides for the exponential amplification reaction of at least one segment of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2), in which the segment encodes a functional portion or a gene-regulating portion of the subunit; - a control DNA from a non affected individual. In a preferred form, the oligonucleotide pairs for the amplification reaction are able to amplify the

entire encoding region of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2).

A further object of the invention is the alpha 2 subunit protein of the Na,K human pump (ATPase, ATP1A2) or a functional portion thereof for use in the diagnosis of pathologies associated with migraine or with alternating hemiplegia of the childhood.

A further object of the invention is the alpha 2 subunit protein of the Na,K human pump (ATPase, ATP1A2) or a functional portion thereof for use in the treatment of pathologies associated with migraine or with alternating hemiplegia of the childhood.

A further object of the invention is a method for the identification of an agonist or antagonist agent of the Na,K human pump (ATPase, ATP1A2) or a functional portion or a gene-regulating portion of thereof, that comprises:

(i) transfection of a cell line with a gene for a mutant isoform of the Na,K human pump (ATPase, ATP1A2) resistant to oubain;

(ii) appropriate exposure of the transfected cells to the agent;

(iii) measurement of the Na,K pump activity in relation to ion transport with labelled ions.

A further object of the invention is a method to identify an agonist or antagonist agent of the Na, K pump (ATPase, ATP1A2) or a functional portion, that comprises the phases:

(i) use of an agent to treat a transgenic animal that expresses a mutant isoform of the Na,K pump (ATPase, ATP1A2) or that partially or completely deletes the gene encoding the Na,K pump (ATPase, ATP1A2) or

(ii) use of an agent to treat eukaryotic or prokaryotic cell lines that express mutant or normal forms of the Na,K pump (ATPase, ATP1A2) by transient or stable transfection or in physiological conditions.

The invention is described below in reference to non limiting examples and the following figures:

Figure 1. *ATP1A2* mutation detection. Panel a, the normal (blue) and mutant (red) D-HPLC elution patterns of exon 17 (left) and 19 (right); Panel b shows

the direct- sequencing electropherograms of the control (upper part) and mutant heterozygotes (lower part); Panel c, the pedigree of the two FHM2 families.

Figure 2. Local amino acid sequence alignment of ATPases. The complete conservation of L764 (left) and W887 (right) in several subunits of Na,K ATPases and H,K ATPases is shown. The relative SwissProt accession number is indicated.

Figure 3. ATP1A2 protein topology. The ouabain binding site on the first loop (M1-M2; asterisks indicate the mutagenized amino acids to confer ouabain resistance) and the two mutations on the largest intracellular (M4-M5) and extracellular (M7-M8) loops are highlighted.

Figure 4. Ouabain treatment of transfected HeLa cells. Phase contrast pictures of HeLa cells taken after 36 hr of 1 μM ouabain challenge transfected with: panel a, mock transfected cells (a construct expressing wild-type ATP1A2 non-ouabain resistant was used); panel b, a ouabain resistant wild-type ATP1A2, pA2Oua^r-wt; panels *d* and f, ouabain resistant ATP1A2 mutants, pA2Oua^r-P764 and pA2Oua^r-R887, respectively; panel c, a 1:1 mixture of pA2Oua^r-wt + pA2Oua^r-P764, to simulate the L764P heterozygous state; panel e, a 1:1 mixture of pA2Oua^r-wt + pA2Oua^r-R887, to simulate the W887R heterozygous state. All experiments were performed by co-transfecting an ATP1B2 expressing construct.

Figure 5. Time course of ouabain toxicity. Panel a, cell viability by MTT assay of HeLa cells transfected with different constructs as reported in Figure 4: mock; A2-wt (pA2Oua^r-wt); mu-1 (pA2Oua^r-P764); het-1 (pA2Oua^r-wt + pA2Oua^r-P764); mu-2 (pA2Oua^r-R887); het-2 (pA2Oua^r-wt + pA2Oua^r-R887). Both mutants and simulated heterozygotes are significantly different from A2-wt (at least $P< 0.04$). Bars represent SD. Panel b, *in vitro* transcription and translation confirming the expected molecular mass of ATP1A2 protein of 112 kDa.

Figure 6. Localization of mutant ATP1A2 to the plasma membrane. Panel a, immunocytochemistry on COS-7 cells of the *c-myc*-derivatives, pA2Oua^r-*wt*-

myc, pA2Oua^r-P764-*myc* and pA2Oua^r-R887-*myc*, showing the plasma membrane localization of both wild type and mutant isoforms. Panel b, subcellular fractionation of transfected COS-7 cells demonstrating the plasma membrane co-sedimentation with ATP1A2 *c-myc*-derivatives; s/n, supernatant; p, pellet.

Figure 7. Phase-contrast pictures of transfected HeLa cells taken after 72h of treatment with 1 μM ouabain. a, transfection with a cDNA construct expressing non-ouabain-resistant wild-type ATP1A2. b, transfection with a cDNA construct expressing ouabain-resistant wild-type ATP1A2. c, transfection with a cDNA construct expressing ouabain-resistant T328N ATP1A2 mutant. d, transfection with a 1:1 mix of constructs expressing ouabain-resistant wild-type ATP1A2 and T328N ATP1A2 mutant to simulate the hetrozygous state.

Figure 8. Cell viability assessed by MTT assay of the transfected HeLa cells shown in Fig. 7. Y axis represents the percentage of surviving cells

EXAMPLE 1 Migraine

Materials and Methods

FHM2 families

Twenty-two subjects from a large Italian pedigree (family 1), originating from Tuscany, with a clinical diagnosis of FHM (5) and seven members with similar manifestations from an unrelated pedigree (family 2) from Sicily, were selected. No cerebellar signs were associated with FHM. The onset of attacks always occurred by the age of twenty. Additional features were history of seizures in five members (three subjects from family 1 and two subjects from family 2) and mild or moderate mental retardation in two subjects from family 1. Two hundred randomly collected healthy individuals from the Italian population were used as control subjects.

Mutation screening

We determined the genomic organization of the human *ATP1A2* gene by aligning the sequence of *ATP1A2* mRNA (AC NM_000702) with the corresponding genomic sequence on clone RP11-536C5. Designed

oligonucleotide primers for amplification of the gene encoding regions are reported in Table 1. PCR products that showed an abnormal D-HPLC (Wave, Transgenomic, Crewe, UK) retention patterns were subjected to direct sequencing (DYEnamic ET Dye Terminator Kit, Amersham Biosciences, Piscataway, NJ, USA).

Table 1

Exon	forward primer	reverse primer	bp
1	TGTTGCTTTGGCTTTCTCTGT	CTCCCTCACCCTCTAGACTGC	177
2+3	CCCCTCTCTTCCCTGACTCT	GCCTCTTTTGTTCCTTCCCTA	423
4	ATGGTGACTGGCTGGGTTG	CAGGGTTGGAGGACAGTCAC	316
5	AGCTGCCCCTTTAGGGTTG	ACCTTACAGCCTAGCCCAGAG	213
6	GAGACCAGCAGGAGAAGAAGG	AGACTCAACTGCTTGCTCTGG	238
7	TACAAGTGGCTCTGCCAGTCT	AGCCCTTCATCCTGACTATGG	234
8	CAGGAAATAGGATGGGACTGC	GTAGTGAGACCCTCCCCTGGT	385
9	ATCTCCGGCTTCAGCCTTAAC	TAATCCTATCCACCCCCTCTG	283
10+11	CTCCTGGTTCCCCCTCAT	TCCCTCTCTCTTCCTCTGTCC	487
12	GCGCTACCAAGACAAGTATGG	CTTGGGAATCCCCTTCTGAG	284
13	GAAGCCACTCTGCGGATCT	ACTGCAGCTCCTTGAACTCTG	286
14	GGAGGGGGATAAACCCTTAAT	GACGTGTTGATTAGGGCACAG	236
15	AGGGGTCAGCTGTCTCTGTC	GGTCCCTGCCTGTCATCTG	284
16	AAGGGGTTTCGTCCTCAAGT	TCAGTATCCTGCAAACCATCC	284
17*	AGTCCCTCTGACCTCCCTGAT	CCACTGTGCCATCACGATT	252
18	TCATCTCCTACGTCCCTTCAA	AGCTGGGAAAAGAACCCTGT	234
19*	CTTCTGCTTCCTGCTCTGACC	ACACATGTGCGCTGTGTTTAC	232
20	CCTCCGACACTCTCATCTGTC	CTGTGTGGGTTGGTGAGTGT	236
21	CTTCACCTGCCACCTCCTT	CCCCCGTATGACTACTCAGG	176
22	CGCTTTGAATGCTCCTTTATG	GAGGGAGGAGCTGGTGGT	223
23	GCCTCCTTTTAAGCTCATGCT	GCCTCATTATCTCTCCCCAAA	206

The primer pairs 17 and 19 (*) were used to identify the two mutations associated with FHM2. The PCR were designed with a uniform annealing temperature of 57° C.

The oligonucleotides that permit the amplification of the gene expression regulating regions (about 3 kb, subdivided in two partially overlapping segment) are listed in Table 2.

Table 2

1_Pr	TTCCCCTCACTCCATCTCTG	GACCCCTGCTCTTTAGGGATA
2_Pr	GATTCAGGACCACTCCATCC	GGGAACAGTCAGAGGACAGG

The analysis of the amplified DNA was performed using direct sequencing and DHPLC (denaturing high-pressure chromatography (16).

Constructs and site-directed mutagenesis

The full-length cDNA coding for the beta 2 (ATP1B2, NM_001678) and alpha 2 were derived from IMAGE clone 23453 and DFKZp761D047, respectively, and subcloned in the expression vector pcDNA3.1 (Invitrogene, Carlsbad, CA, USA). We used the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) for mutagenizing the *ATP1A2* cDNA as follows:

a) nt451 A>G (Q116R) and nt483 A>G (N127D) to obtain the construct pA2Oua^r-wt expressing the ouabain-resistant isoform;

b) nt2395 T>C (L764P) on pA2Oua^r-wt, obtaining pA2Oua^r-P764;

c) nt2763 T>C (W887R) on pA2Oua^r-wt, obtaining pA2Oua^r-R887.

d) *c-myc*-tagging the *ATP1A2* cDNA. Expression constructs (pA2Oua^r-wt, pA2Oua^r-P764, and pA2Oua^r-R887) were mutagenized by replacing the original start codon with the *c-myc* tag (consisting of aa MAEEQKLISEEDL, corresponding to aa 408-419 of the human c-myc AC 0907235A) obtaining pA2Oua^r-wt-*myc*, pA2Oua^r-P764-*myc* and pA2Oua^r-R887-*myc*. All constructs were sequence-verified.

In vitro transcription and translation

In vitro transcription and translation was performed using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) in the presence of 20 microCi [^{35}S] methionine (1000 Ci/mmole) and neosynthesized proteins were separated by SDS/PAGE (8%).

Electrophoresis and western blot analysis

Equal amounts of proteins were resuspended in SDS-PAGE buffer (62.5 mM Tris- HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and separated for 2 h at 100V in 10% SDS-polyacrylamide gels. Transblotted nitrocellulose membranes were incubated with monoclonal primary antibodies anti c-*myc* 9E10 (10 µg/ml) or polyclonal antibodies anti-Integrin beta 1 followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized with the Enhanced Chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Transfections and ouabain treatment

Constructs were transfected by calcium phosphate (HeLa cells) following standard procedures or by Lipofectamine (COS-7 cells), (Gibco, Invitrogene Co., Carlsbad, CA, USA).

Cell viability was measured with the MTT (13) reduction assay after 12, 24, 36, 48 and 60 hours. Each experiment was made in triplicate and statistical analysis was done by Student's *t*-test (homoscedastic).

Immunocytochemistry

After transfection (48 hr), COS-7cells were fixed in 100% methanol and incubated with monoclonal primary antibodies anti c-*myc* 9E10. Cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA). Cells were coverslipped in fluorescent mounting medium (DAKO, Glostrup, Danmark) and visualized under epifluorescence optics.

Subcellular fractionation

COS-7 cells were lysated on ice in 0.5 M NaCl, 10 mM NA2C03, 0.1 mM PMSF, 10 µg/ml Aprotenin, 10 µg/ml Leupeptin, homogenated and centrifuged at 2000 g for 20 min at 4°C to discard nuclei and cellular debris. Separation of the membrane fraction (pellet) from the cytosolic fraction (supernatant) was achieved by centrifugation at 100,000 g for 40 min at 4°C in a Beckman TL 100 ultracentrifuge.

Results

Mutation analysis

Although the reduced FHM2 critical region spanned only 0.9 Mb between D1S2635 and CASQ1-SNP, several positional candidate genes, which are expressed in the central nervous system, are present in this genomic area. We performed mutation analysis on two probands of the FHM2 families by both D-HPLC (denaturing HPLC) and direct sequencing on the two potassium channel genes, *KCNJ9* and *KCNJ10* and the *CASQ1* gene coding for calsequestrin with negative results.

In contrast, D-HPLC mutation scanning of the *ATP1A2* gene encoding the alpha 2 subunit of the Na,K ATPase gave aberrant elution patterns for exon 17 and exon 19 in families 1 and 2, respectively (Fig. 1a). Sequencing analysis revealed the presence of two point mutations (nt 2395 T to C and nt 2763 T to C; Fig. 1b), each segregating with the disease in the respective families (Fig.1c) and causing the amino acid replacements leucine to proline (L764P) in family 1 and tryptophan to arginine (W887R) in family 2. Both missense mutations were absent in 400 control chromosomes. L764 and W887 are completely conserved among alpha subunits from several evolutionary distant species (Fig. 2), thus strongly suggesting a causal role of ATP1A2 mutations in the pathogenesis of FHM2.

The Na,K pump is a heterodimeric structure formed by a large catalytic alpha subunit and a small ancillary beta subunit. The alpha subunits traverse the plasma membrane with 10 transmembrane segments (M1-M10) (14) and expose the amino- and carboxy-termini towards the cytoplasm. This configuration assigns five extracellular and four intracellular loop domains. L764P and W887R mutations have different localization within the alpha topology: L764P maps to the large intracellular loop between M4 and M5, while W887R localizes to the apical M7-M8 loop (Fig. 3).

Functional evidence of impaired ion transport

In order to evaluate the functional consequences of these two amino acid replacements involving different protein structures, we carried out various transfection experiments. We cloned the full-length human cDNAs of the

alpha 2 and beta 2 subunits (see Materials and Methods section). Since both subunits are required for assembling the active alpha-beta heterocomplex, all transfection experiments hereafter were carried out by co-transfecting alpha 2 and beta 2 constructs with equal stoichiometry. By introducing the mutant isoforms (i.e. expressing the mutant P764 and R887 full-length cDNAs), we obtained no significant changes in the cell shape or growth rate (data not shown), thus excluding a primary dominant-negative effect.

As all vertebrate cells present Na,K ATPase activity, the endogenous Na,K pump activity of HeLa cells was quenched to test the ion transport performance of the exogenous mutant forms. A site-directed mutagenesis was carried out to abolish the natural ouabain sensitivity of the ATP1A2 construct by mutagenizing two amino acids, Q116R and N127D, in the first extracellular loop that is part of the ouabain binding site of Na,K ATPase (15). HeLa cells transfected with the ouabain-resistant *ATP1A2* cDNA construct (pA2Oua^r-wt) can survive and grow in 1 μM ouabain-containing media (Fig. 4, panel b), while mock transfected cells die within 36--48 hours (Fig. 4, panel a). Identical results were obtained with the human cell line HEK293.

Once positively tested for ouabain resistance, the pA2Oua^r-wt construct was subsequently mutagenized to introduce the FHM2 mutations L764P and W887R, obtaining the corresponding constructs pA2Oua^r-P764 and pA2Oua^r-R887. HeLa cells transfected with pA2Oua^r-P764 or pA2Oua^r-R887 failed to survive 1 μM ouabain treatment (Fig. 4, panels *d* and f), thus suggesting that both L764P and W887R are loss-of-function mutations.

Simulated heterozygous states obtained by co-transfecting equal amounts of wild-type and mutant constructs showed an intermediate behavior (Fig. 4, panel *c* and e). Both mutant ATP1A2 isoforms showed early cell mortality typical of cells lacking Na,K pump activity (Fig. 5a). To exclude the possible production of aberrant proteins from site-directed mutagenesis, we tested the wt and mutant constructs by *in vitro* transcription and translation experiments and direct sequencing. As shown in Figure 5b, all three constructs gave the expected 112 kDa protein band, thus excluding the possibility that a cloning

artifact is responsible for the vulnerability of HeLa cells to ouabain when transfected with the mutant *ATP1A2* cDNAs.

Mutant ATP1A2 isoforms are delivered to the plasma membrane

We investigated the subcellular localization of the two mutant isoforms. The three constructs (pA2Oua^r-wt, pA2Oua^r-P764, and pA2Oua^r-R887) were engineered by adding a 5' tag coding for the *c-myc* epitope and transfected into COS-7 cells. Figure 6a shows the expected immunofluorescence localization to the plasma membrane of all isoforms, both wild type and the two mutants. Subcellular fractionation confirmed the physiological location in the membrane fraction (Fig. 6b), where the integrin beta 1 subunit was detected as a control.

These data demonstrate that both missense mutations are independently sufficient to inhibit Na,K pump activity, without affecting the assembling with the beta subunit and the complex translocation to the cell membrane.

Example 2 Alternating hemiplegia of the childhood.

Materials and Methods

Constructs and site-directed mutagenesis.

The full length cDNA coding for the alpha 2 was subcloned in the expression vector pcDNA3.1 (Invitrogene, Carlsbad, CA, USA). We used the QuickChange site-directed mutagenesis kit (Stratagene, La Iolla, CA, USA) for mutagenizing the *ATP1A2* cDNA as follows:

Q116R and N127D to obtain the construct pA2Oua^r-wt expressing the ouabain resistant isoform;

T378N on pA2Oua^r-wt, obtaining pA2Oua^r-AHC mutated form;

All constructs were sequence-verified.

Transfections and ouabain treatment.

Constructs were transfected by calcium phosphate (HeLa cells) following standard procedures.

Cell viability was measured with the MTT reduction assay after 24, 48 and 72 hours of 1 µM ouabain challenge. We performed two independent experiments and in each experiment, datapoints are in triplicate.

Results

Alternating hemiplegia of the childhood (AHC, OMIM 104290) is a rare syndrome (estimated prevalence 1 in 1.000.000), characterized by early onset of episodic hemi- or quadriplegia lasting minutes to days.

Mutation analysis in the ATP1A2 gene was performed by direct sequencing of all exons with the same primers used for amplification. An heterozygous mutation (1237 C->A) segregating with the disease in a AHC family and causing a threonine to asparagine replacement (T378N) was found. This mutation is not present in any of the unaffected members of the family and in 250 control chromosomes.

This missense mutation localizes to the ATPases phosphorylation site (DKTGTLT, aa 374-380) of the hydrolase domain of the protein. In the □□ subunit topology the mutated residue resides in the large intracellular loop within the M4-M5 transmembrane segments (M1-M10, [Hu, 2000 #7823]). The affected residue is highly conserved in all the known □ subunits of the Na,K pump from vertebrates to invertebrates suggesting a functional role in pump activation.

To evaluate the functional consequences of this mutation we carried out transfection experiments in human HeLa cells. Since all mammalian cells have Na+/K+ ATPase, we quenced the endogenous pump activity using ouabain, and tested the function of the exogenously transfected mutant of cDNA constructs engineered to be resistant to ouabain. Using site-directed mutagenesis we introduced two amino acid changes (Q116R and N127D) in the first extracellular loop to confer resistance to ouabain, and the AHC mutation T378N. HeLa cells transfected with this construct did not survive to1 μM ouabain treatment. A simulated heterozygous state, as obtained by transfecting equal amount of wild-type and mutant cDNAs, showed intermediate behaviour (Fig. 7).

In addition, as revealed by time course experiments, the mutant isoform show rapid mortality typical of cells lacking Na^+/K^+ ATPase pump activity (Fig. 8).

References

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CLAIMS

1. Nucleic acid comprising at least one segment of the gene encoding a functional portion or the gene-regulating region of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in the diagnosis of pathologies associated with migraine or with alternating hemiplegia of the childhood.

2. Nucleic acid comprising at least one segment of the gene encoding a functional portion or the gene-regulating region of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in genetic therapy for pathologies associated with migraine or with alternating hemiplegia of the childhood.

3. Method to detect in an individual at least one mutation in the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) located on chromosome1, associated with migraine disorders, which comprises the steps of:

- collecting a sample containing a sufficient quantity of the individual's DNA or that is reproducible in culture;
- isolating of the DNA from the sample;
- exponential amplifying the DNA using as an oligonucleotide pair for the amplification reaction at least two oligonucleotides that are able to amplify at least one segment of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) or a segment of the region regulating it;
- detecting in at least one amplified segment any mutations compared with a healthy control.

4. Method according to claim 3 in which the oligonucleotide pairs are:

17	AGTCCCTCTGACCTCCCTGAT	CCACTGTGCCATCACGATT
19	CTTCTGCTTCCTGCTCTGACC	ACACATGTGCGCTGTGTTTAC.

5. Method according to claim 3 in which the DNA exponential amplification phase is performed using oligonucleotide pairs that are able to amplify the entire encoding portion of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2).

6. Method according to claim 5 in which the DNA exponential amplification phase to amplify the entire portion encoding the gene for the alpha 2 subunit

of the Na,K human pump (ATPase, ATP1A2) comprises the use of at least one of the following oligonucleotide pairs:

1	TGTTGCTTTGGCTTTCTCTGT	CTCCCTCACCCTCTAGACTGC
2+3	CCCCTCTCTTCCCTGACTCT	GCCTCTTTTGTTCCTTCCCTA
4	ATGGTGACTGGCTGGGTTG	CAGGGTTGGAGGACAGTCAC
5	AGCTGCCCCTTTAGGGTTG	ACCTTACAGCCTAGCCCAGAG
6	GAGACCAGCAGGAGAAGAAGG	AGACTCAACTGCTTGCTCTGG
7	TACAAGTGGCTCTGCCAGTCT	AGCCCTTCATCCTGACTATGG
8	CAGGAAATAGGATGGGACTGC	GTAGTGAGACCCTCCCCTGGT
9	ATCTCCGGCTTCAGCCTTAAC	TAATCCTATCCACCCCCTCTG
10+11	CTCCTGGTTCCCCCTCAT	TCCCTCTCTCTTCCTCTGTCC
12	GCGCTACCAAGACAAGTATGG	CTTGGGAATCCCCTTCTGAG
13	GAAGCCACTCTGCGGATCT	ACTGCAGCTCCTTGAACTCTG
14	GGAGGGGGATAAACCCTTAAT	GACGTGTTGATTAGGGCACAG
15	AGGGGTCAGCTGTCTCTGTC	GGTCCCTGCCTGTCATCTG
16	AAGGGGTTTCGTCCTCAAGT	TCAGTATCCTGCAAACCATCC
17	AGTCCCTCTGACCTCCCTGAT	CCACTGTGCCATCACGATT
18	TCATCTCCTACGTCCCTTCAA	AGCTGGGAAAAGAACCCTGT
19	CTTCTGCTTCCTGCTCTGACC	ACACATGTGCGCTGTGTTTAC
20	CCTCCGACACTCTCATCTGTC	CTGTGTGGGTTGGTGAGTGT
21	CTTCACCTGCCACCTCCTT	CCCCCGTATGACTACTCAGG
22	CGCTTTGAATGCTCCTTTATG	GAGGGAGGAGCTGGTGGT
23	GCCTCCTTTTAAGCTCATGCT	GCCTCATTATCTCTCCCCAAA

7. Method according to claim 3 in which the DNA exponential amplification phase is performed using oligonucleotide pairs that are able to amplify the regulating region of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2).

8. Method according to claim 7 in which the DNA exponential amplification phase to amplify the regulating region of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2) comprises the use of the following oligonucleotide pairs:

1_Pr	TTCCCCTCACTCCATCTCTG	GACCCCTGCTCTTTAGGGATA
2_Pr	GATTCAGGACCACTCCATCC	GGGAACAGTCAGAGGACAGG.

9. Method according to the aforementioned claims in which the detection phase of at least one amplified segment with any mutations compared with a healthy control is performed using direct sequencing or an SSCP method (single strand conformation polymorphism) (17) DHPLC or DGGE (denaturing gradient gel electrophoresis) (18).

10. Diagnostic kit for pathologies associated with migraine or with alternating hemiplegia of the childhood to carry out the method according to claims 3 through 9, that comprises:

- at least one pair of oligonucleotides for the exponential amplification reaction of at least one segment of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2), in which the aforesaid segment encodes a functional portion or a gene-regulating portion of the aforesaid subunit;
- a control DNA from a non affected individual.

11. Kit according to claim 10 in which the oligonucleotide pairs for the amplification reaction are able to amplify the entire encoding region of the gene encoding the alpha 2 subunit of the Na,K human pump (ATPase, ATP1A2).

12. Alpha 2 subunit protein of the Na,K human pump (ATPase, ATP1A2) or a functional portion thereof for use in the diagnosis of pathologies associated with migraine or with alternating hemiplegia of the childhood.

13. Alpha 2 subunit protein of the Na,K human pump (ATPase, ATP1A2) or a functional portion thereof for use in the treatment of pathologies associated with migraine.

14. Method for the identification of an agonist or antagonist agent of the Na,K human pump (ATPase, ATP1A2) or a functional portion or a gene-regulating portion of the subunit, that comprises:

(i) transfection of a cell line with a gene for a mutant isoform of the Na,K human pump (ATPase, ATP1A2) resistant to ouabain;

(ii) appropriate exposure of the transfected cells to the agent;

(iii) measurement of the Na,K pump activity in relation to ion transport with labeled ions.

15. Method for the identification of an agonist or antagonist agent of the Na,K pump (ATPase, ATP1A2) or a functional portion, that comprises the phases:

(i) use of the agent to treat a transgenic animal that expresses a mutant isoform of the Na,K pump (ATPase, ATP1A2) or that is partially or completely deleted in the gene encoding the Na,K pump (ATPase, ATP1A2) or

(ii) use of the agent to treat eukaryotic or prokaryotic cell lines that express mutant or normal forms of the Na,K pump (ATPase, ATP1A2) by transient or stable transfection or in physiological conditions.

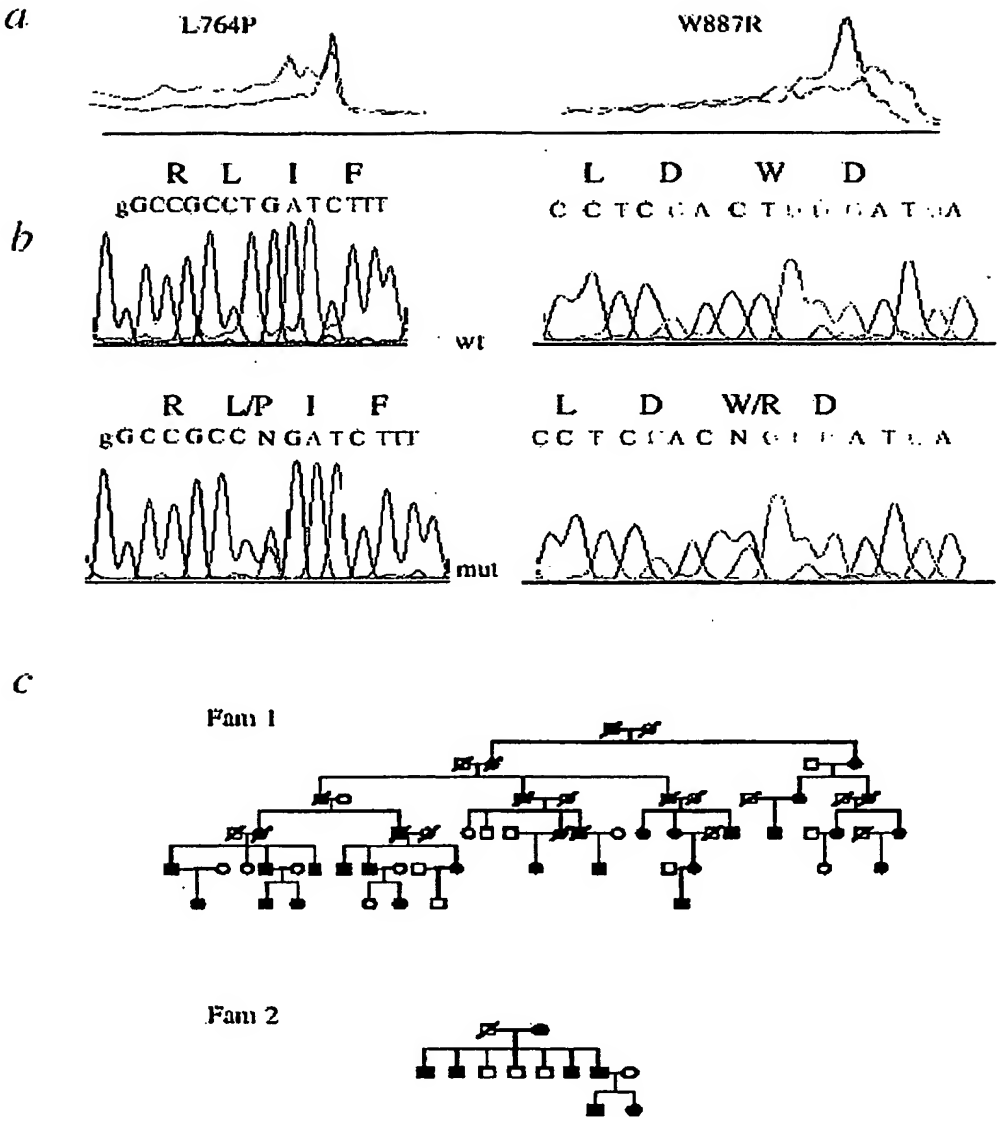
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FIGURE 1

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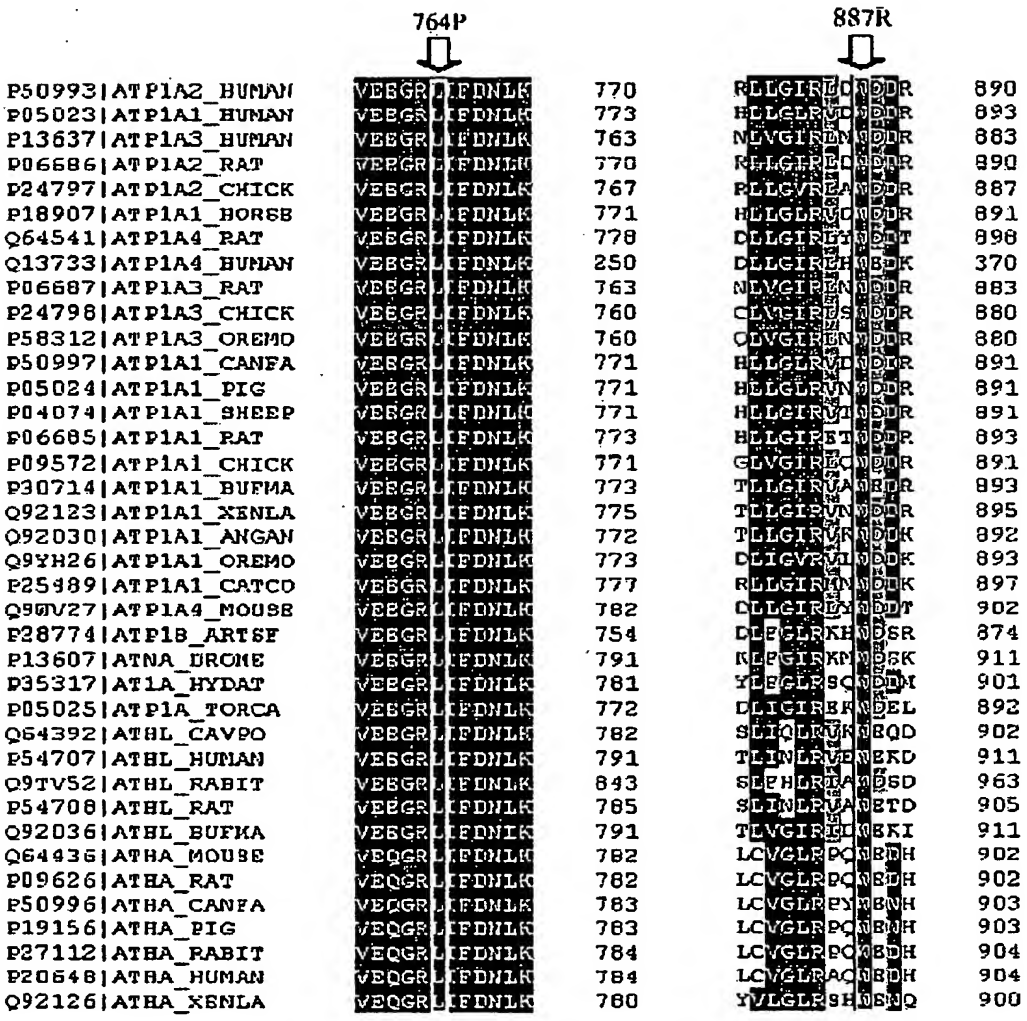
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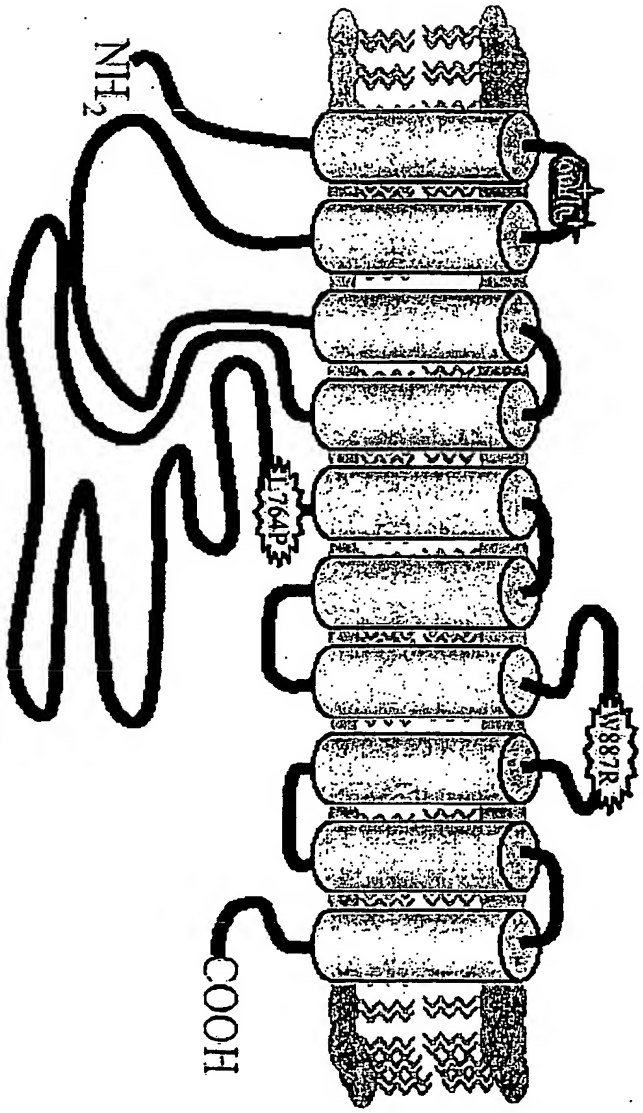
FIGURE 2



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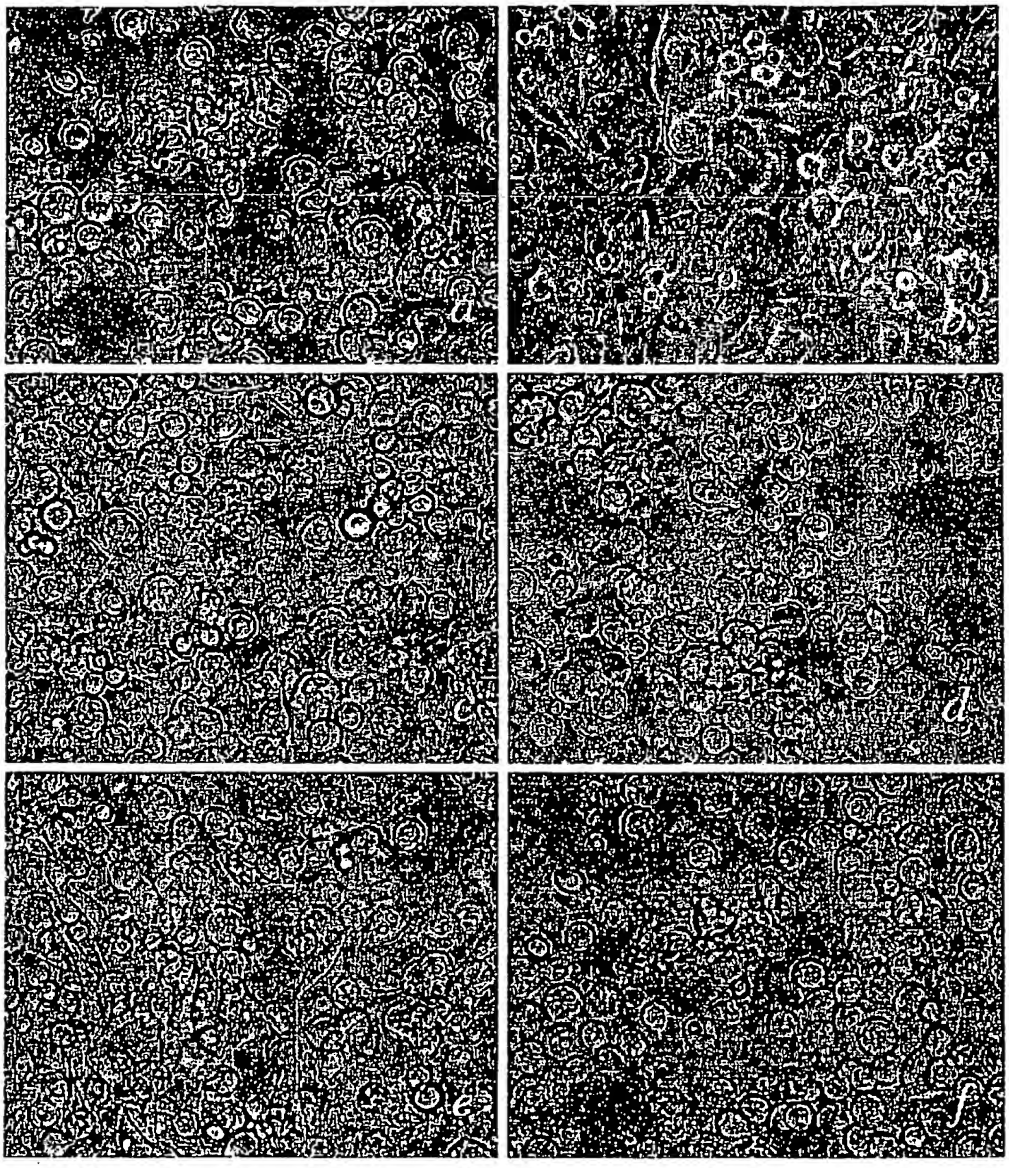

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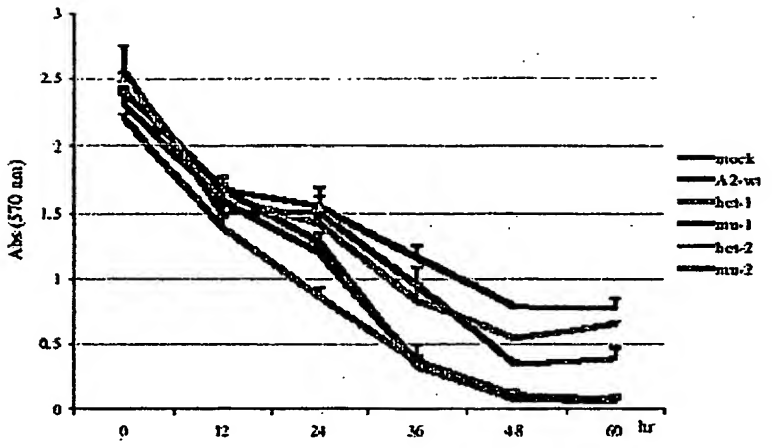
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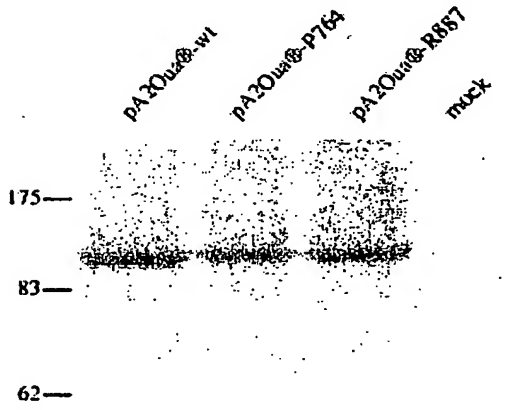
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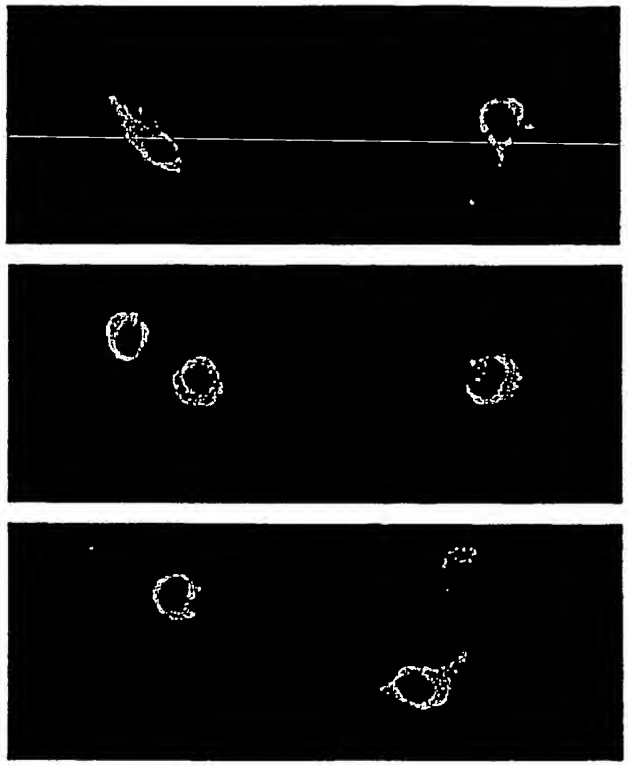
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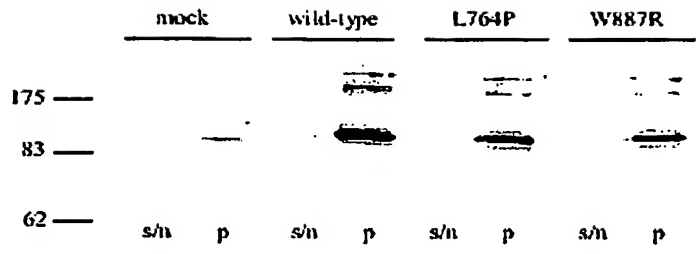
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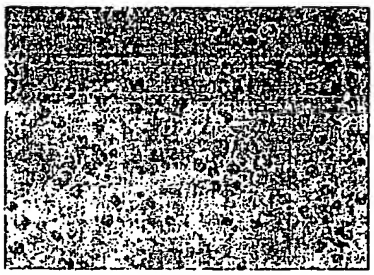
FIGURE 6



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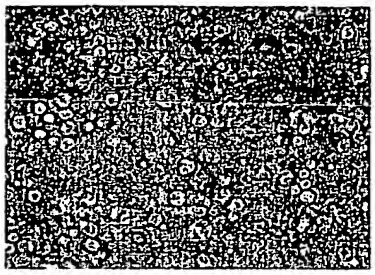

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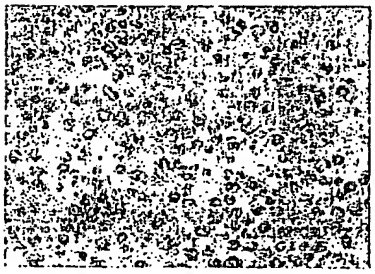

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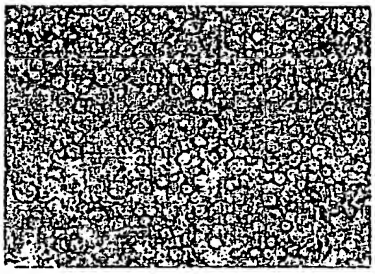

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FIGURE 7

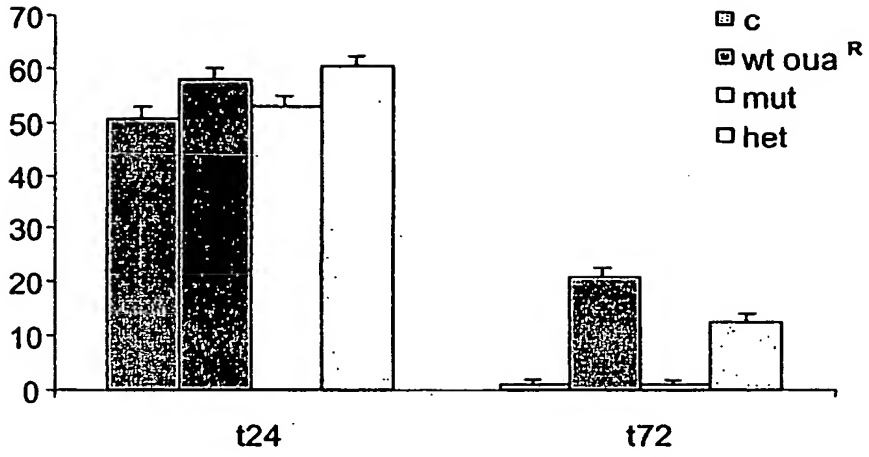

FIGURE 8

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DIAGNOSTIC AND THERAPEUTIC MEANS FOR PATHOLOGIES ASSOCIATED WITH ALPHA 2 SUBUNIT OF THE NA, K PUMP

(57) Abstract: A nucleic acid is described that comprises at least one segment of the gene encoding a functional segment of the alpha 2 subunit of the Na,K pump (ATPase, ATP1A2) for use in the diagnosis or treatment of pathologies associated with migraine or with alternating hemiplegia of the childhood. Appropriate diagnostic kits and methods to identify agonist or antagonist agents are also described.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/12635

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE GENEBANK 19 March 1999 (1999-03-19), LINGREL: "Homo sapiens ATPase, Na,K transporting, alpha 2 (+) polypeptide (ATP1A2), mRNA" XP002283888 Database accession no. NM_000702 abstract -/--	1,2,12, 13

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 2004	23/06/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bort, S

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/12635

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	& SHULL M M ET AL: "CHARACTERIZATION OF THE HUMAN NA,K-ATPASE ALPHA2 GENE AND IDENTIFICATION OF INTRAGENIC RESTRICTION FRAGMENT LENGTH POLYMORPHISMS" JOURNAL OF BIOLOGICAL CHEMISTRY, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.,, US, vol. 264, no. 29, 15 October 1989 (1989-10-15), pages 17532-17543, XP001027129 ISSN: 0021-9258 Characterisation of the NA,K ATPase alpha 2 gene	1,2,12, 13
A	DUCROS A ET AL: "MAPPING OF A SECOND LOCUS FOR FAMILIAL HEMIPLEGIC MIGRAINE TO LQ21-Q23 AND EVIDENCE OF FURTHER HETEROGENEITY" ANNALS OF NEUROLOGY, BOSTON, US, vol. 42, no. 6, 1 December 1997 (1997-12-01), pages 885-890, XP002058761 ISSN: 0364-5134 Linkage analysis studies in families with familial hemiplegic migraine and markers from 1q21-q23	
A	GLENN B S ET AL: "RELATION OF ALLELES OF THE SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATASE ALPHA2 GENE WITH BLOOD PRESSURE AND LEAD EXPOSURE" AMERICAN JOURNAL OF EPIDEMIOLOGY, SCHOOL OF HYGIENE & PUBLIC HEALTH OF THE JOHNS, US, vol. 153, no. 6, 15 March 2001 (2001-03-15), pages 537-545, XP001027407 ISSN: 0002-9262 Association of polymorphisms in the NA,K ATPase alpha 2 gene with blood pressure and lead exposure the whole document	
A	HAAN ET AL: "Alternating hemiplegia of childhood: no mutations in the familial hemiplegic migraine CACNA1A gene" CEPHALALGIA, vol. 20, no. 8, October 2000 (2000-10), pages 696-700, XP002283887 Mutational screening of the CACNA1A gene in cases with alternating hemiplegia of childhood	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/12635

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 3-9, 15
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 3-9 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the sequences.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.